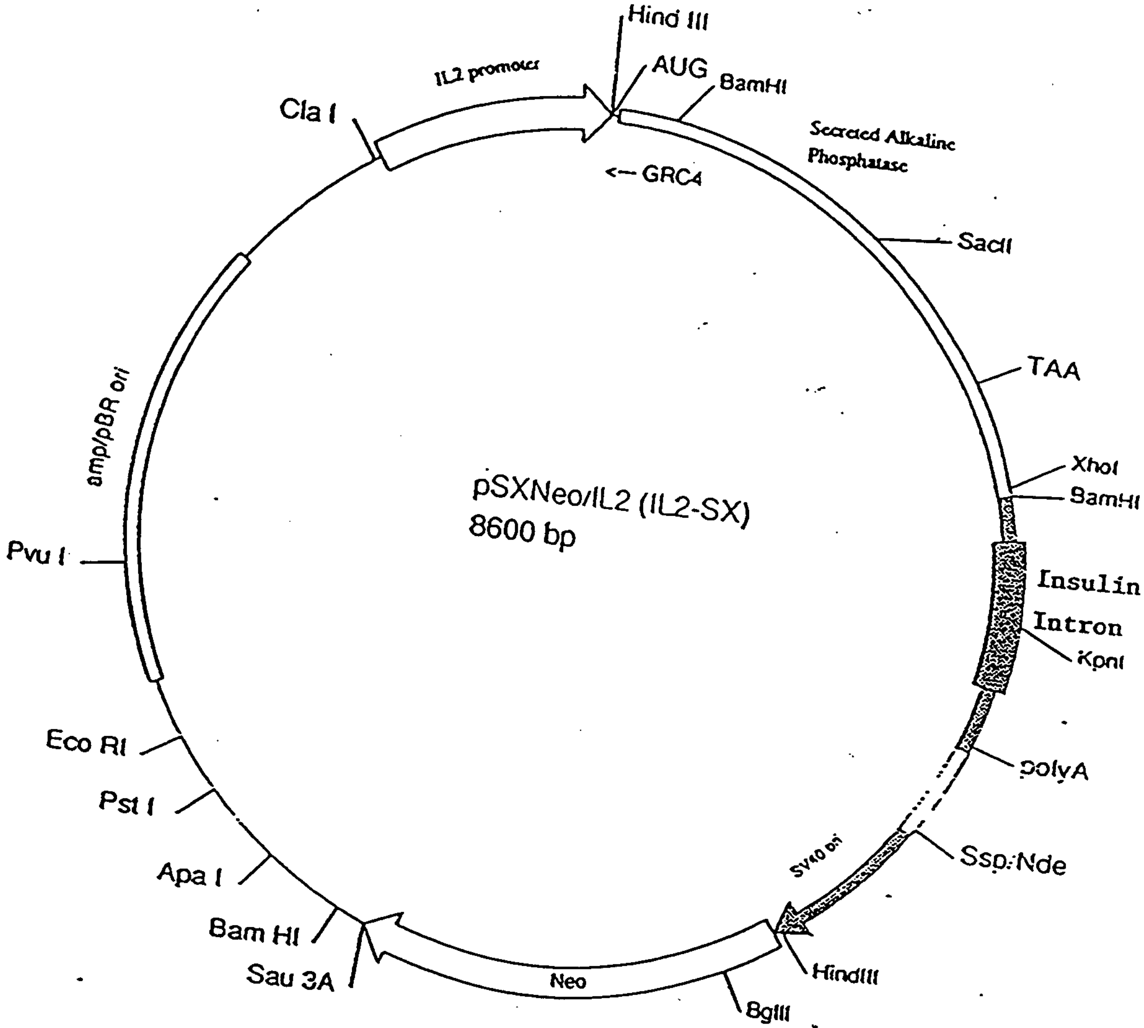
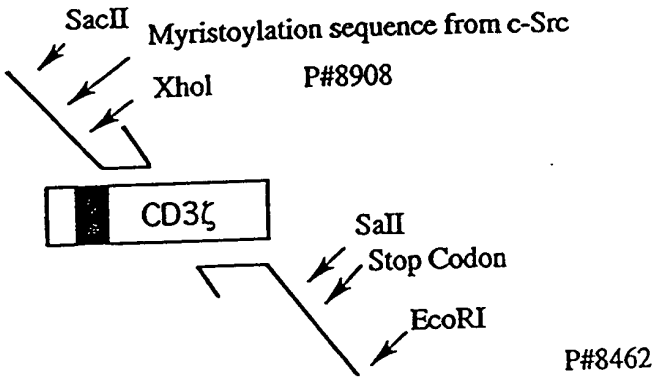
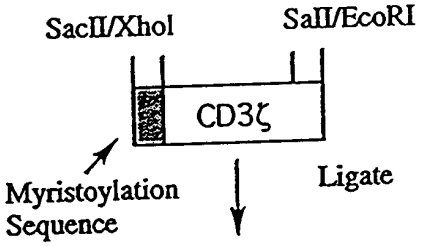


Figure 1/21

Construction of intracellular signalling chimera:

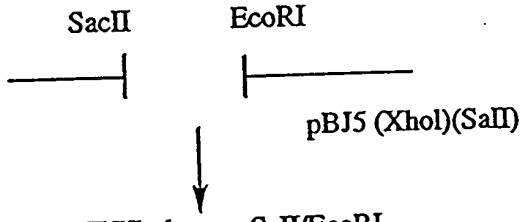
1. PCR myristoylated CD3ζ

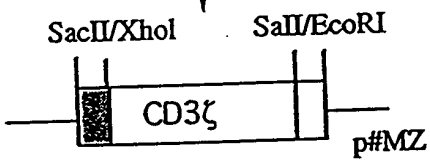


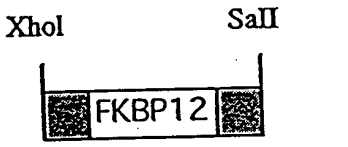


2. Cut and clone PCR fragment

*The MZE series contains a 9aa HA epitope at the 3' end.



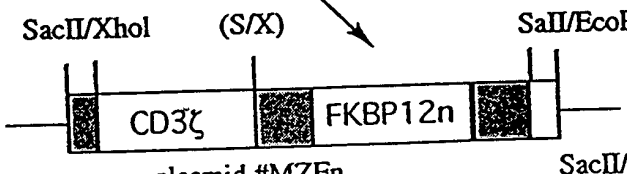




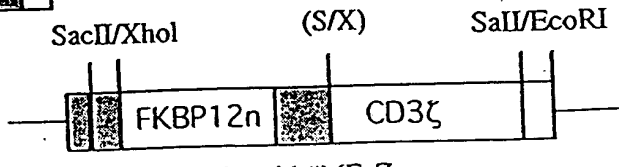
From plasmid #FK12/KS

3. SEQUENCE insert

4. Cut at XhoI or SalI and add FKBP domains



plasmid #MZFn



plasmid #MFnZ

Figure 2/21

Construction of extracellular signaling chimera:

1. PCR murine signal peptide

2. PCR CD3 trans-membrane and cytoplasmic domains

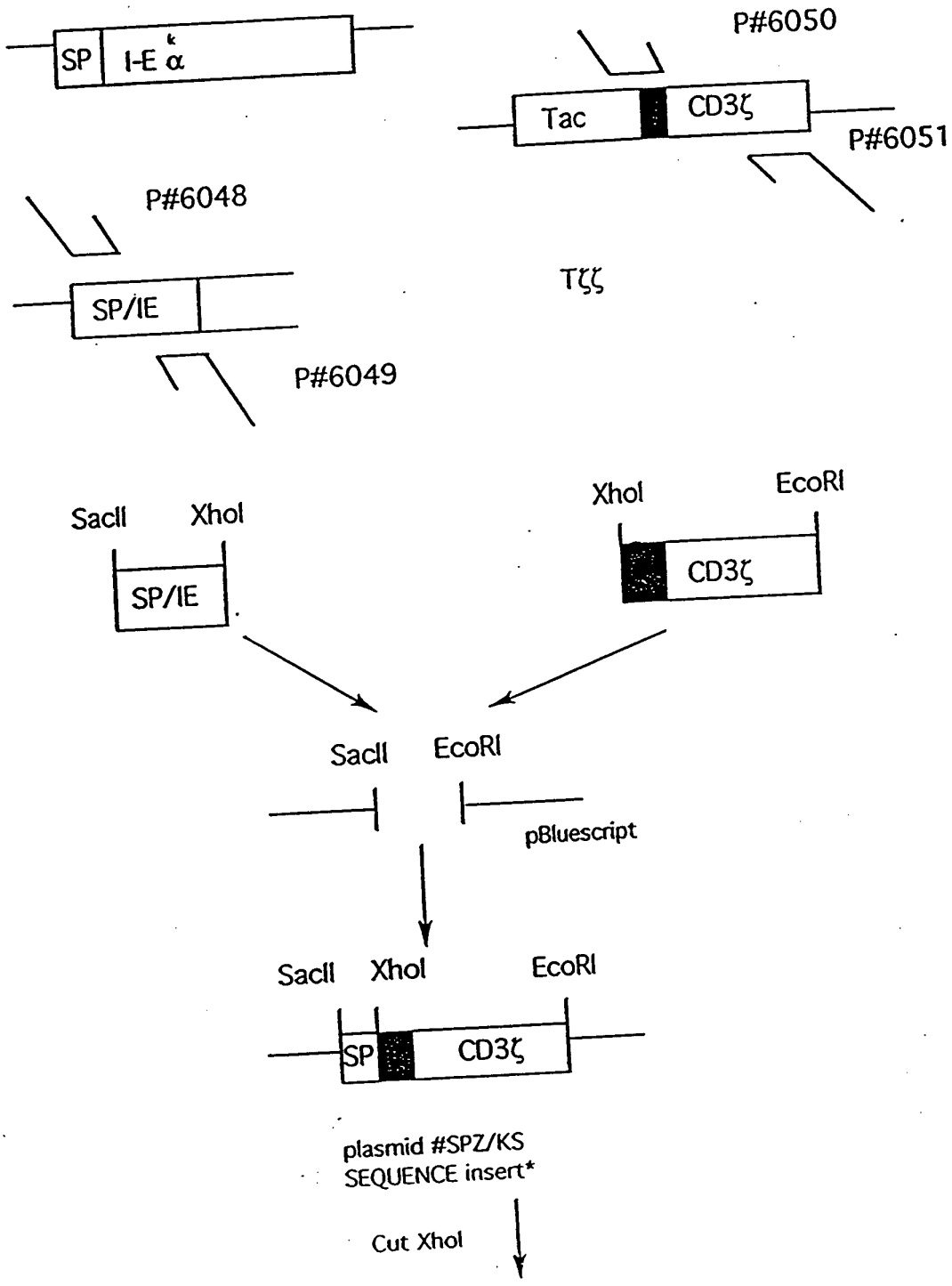


Figure 3A/21

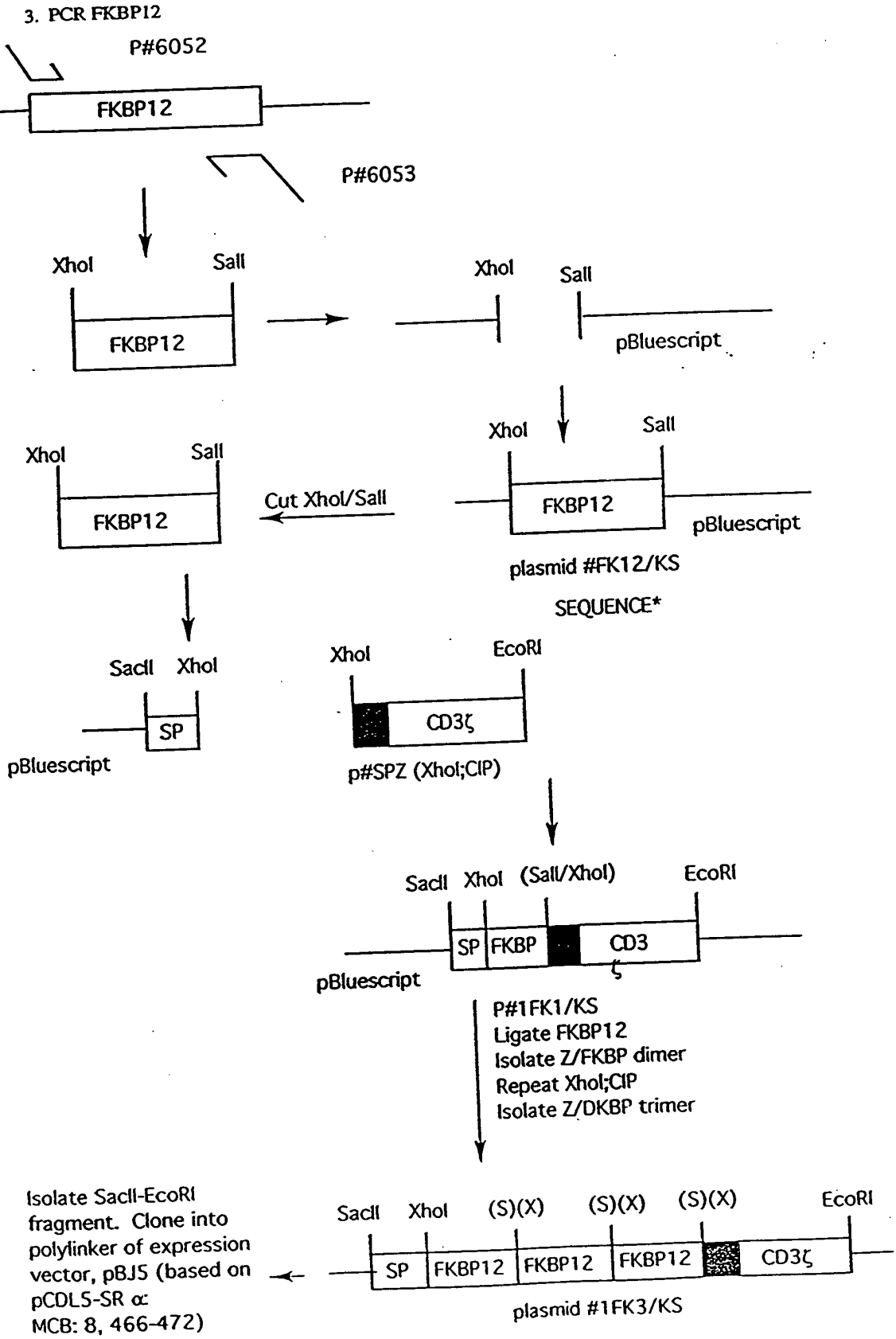
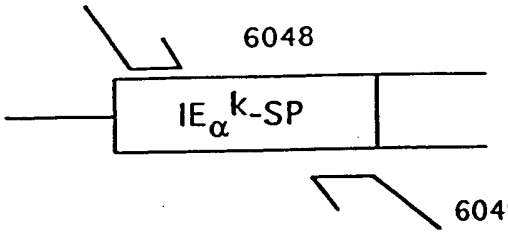


Figure 3B/21



homology

SacII

6048: 5'-CGACACCGCGGCCACCATGGCCACAATTGGAGC-3'

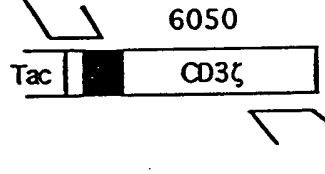
Kozak M A T I G

homology

XhoI

6049: 5'-CGACACTCGAGAGCCCATGACTTCTGG

L A W S _ ?



Asp-Gyl#1

homology

XhoI *

6050: 5'CGACACTCGAGCTCTGCTACTTGCTAGGTGGAATCCTCTTC-3'

E L C Y L L G G I L F

*A to G

homology

EcoRI *

6051: 5' GCGAATTCTTAGCGAGGGGCCAGC-3'

St R P A L *G to C

3'Sal #B

homology

EcoRI SalI

8462: 5' GCGAATTCTTAGTCGACGCGAGGGGCCAGGGTC-3'

St R PAL

Cys-Gly #2

homology

XhoI *

7129: 5' GGGCTCGAGCTCGGCTACTTGCTAG-3'

L G Y L L *T to G

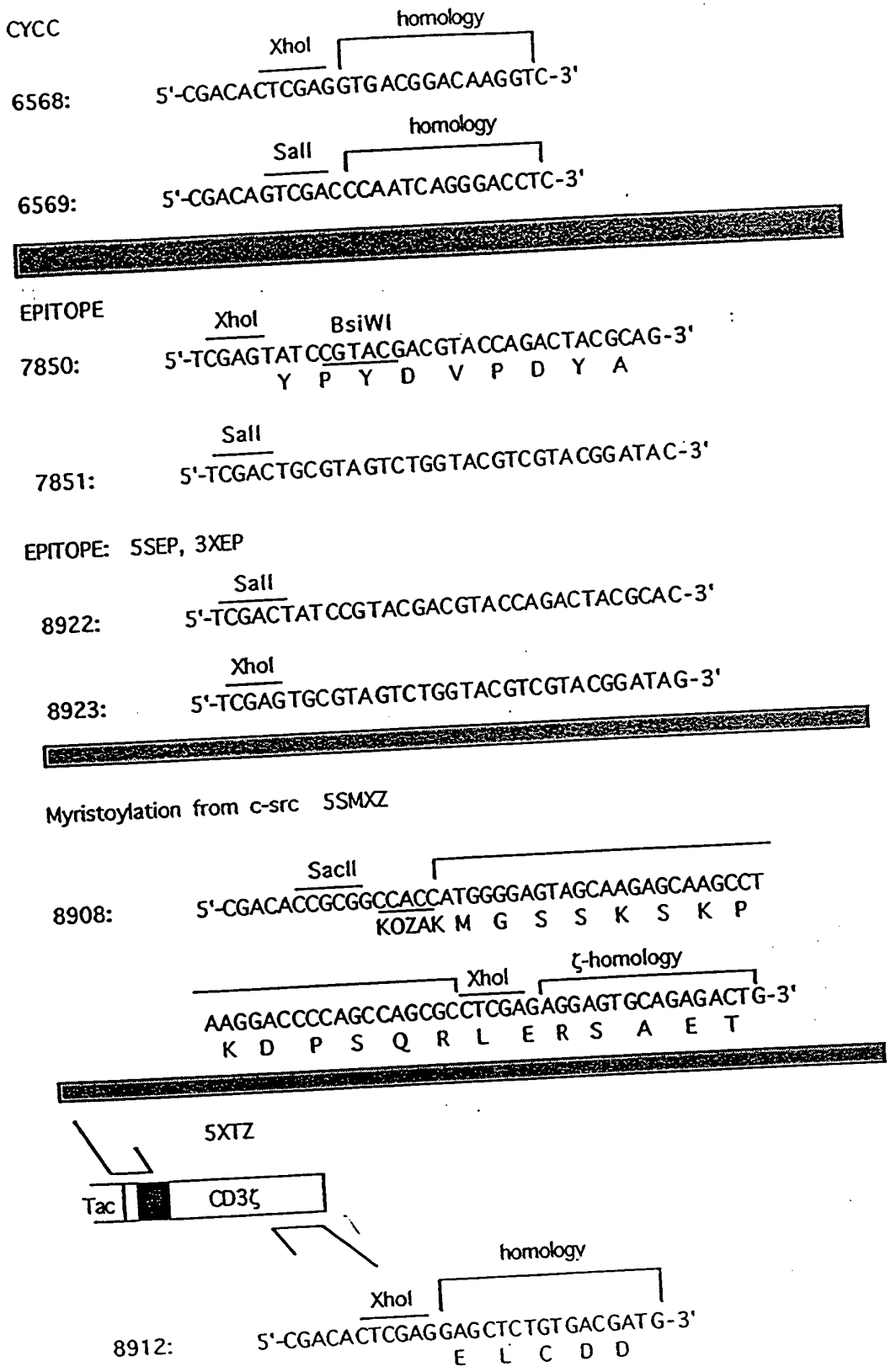


Figure 4B/21

Asp-Lys #4

8061: 5'-CGACA CTCGAG CTCTGCTACTTGCTA AAG GGA ATCCTCTTC-3'
(XhoI site over CTCGAG; bracket marked "homology" over CTCTGCTACTTGCTAAAGGGAATCCTCTTC; * * over AAG)
E L C Y L L K G I L F

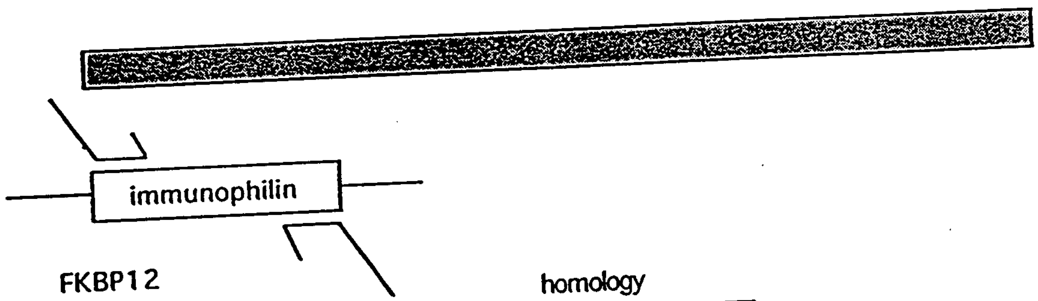
*GATtoAAG

#4 Extension

8907: 5'-CGACA CTCGAG CTGCTGGATCCGAAG CTCTGCTACTTGCTAAAG-3'
(XhoI site over CTCGAG; bracket marked "homology" over CTCTGCTACTTGCTAAAG)
E L L D P K L C Y L L K

TAC-Tm #3

7220: 5'-CGACA CTCGAG ACAACAGAGTACCAGGTAGC-3'
(XhoI site over CTCGAG; bracket marked "homology" over ACAACAGAGTACCAGGTAGC)
E T T E Y Q V



FKBP12

6052: 5'-CGACA CTCGAG GGCGTGCAGGTGGAGAC-3'
(XhoI site over CTCGAG; bracket marked "homology" over GGCGTGCAGGTGGAGAC)
E G V Q V E

6053: 5'-CGACA GTCGAC TTCCAGTTTTAGAAGC-3'
(SalI site over GTCGAC; bracket marked "homology" over TTCCAGTTTTAGAAGC)
V E L K L L

FKBP13

8460: 5'-TCGACA CTCGAG ACGGGGGCCGAGGGC-3'
(XhoI site over CTCGAG; bracket marked "homology" over ACGGGGGCCGAGGGC)
E T G A E G

8461: 5'-CCGACA GTCGAC CTCTATTTTGAGCAGC-3'
(SalI site over GTCGAC; bracket marked "homology" over CTCTATTTTGAGCAGC)
V E I

Figure 4C/21

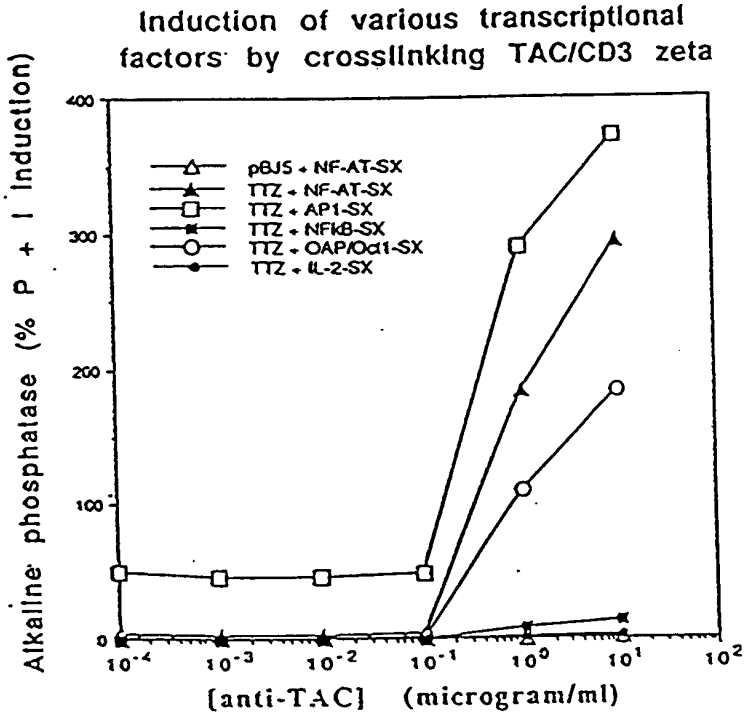


Figure 5/21

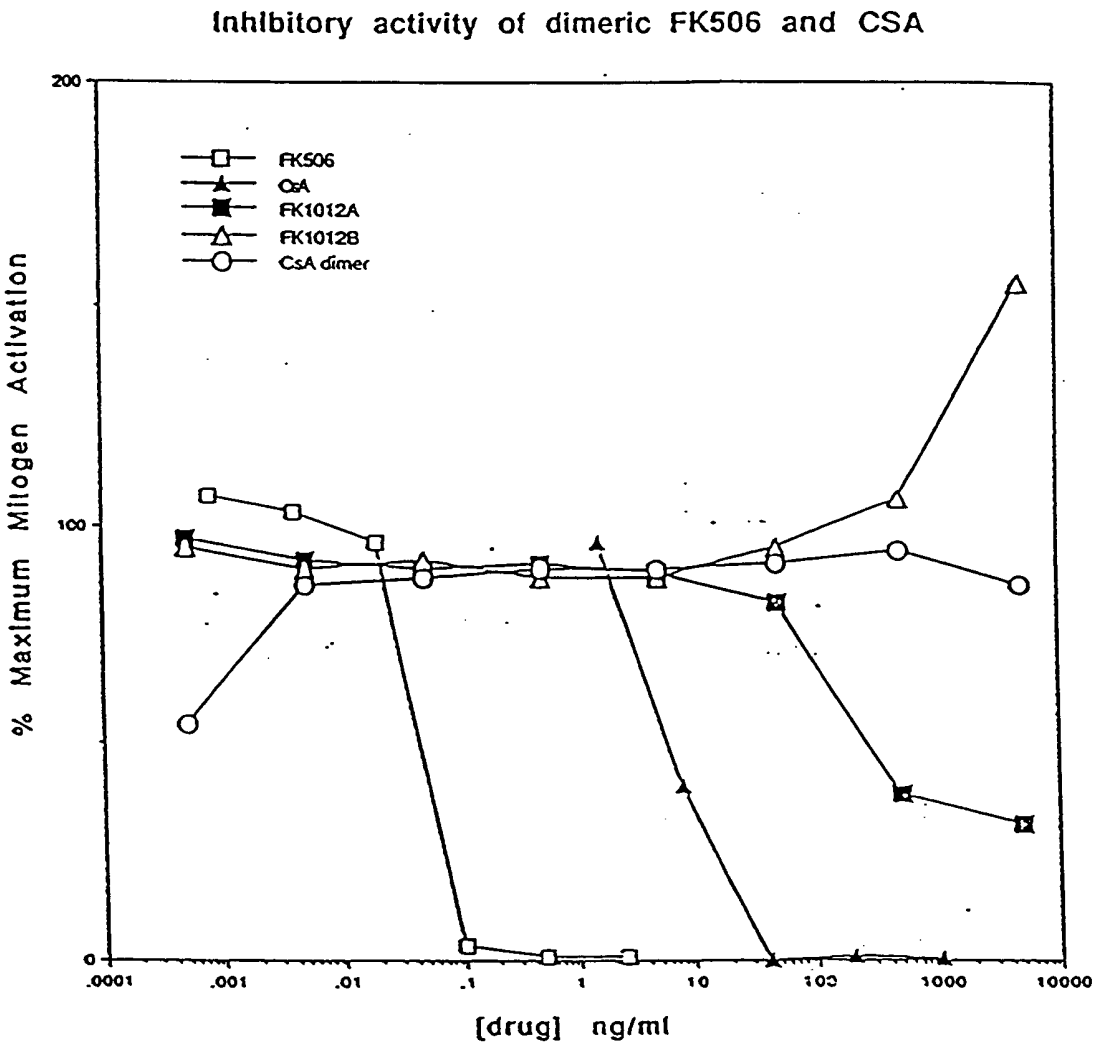
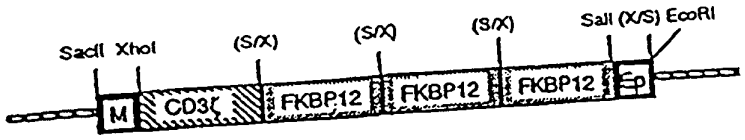
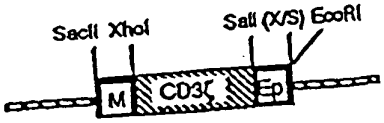


Figure 6A/21

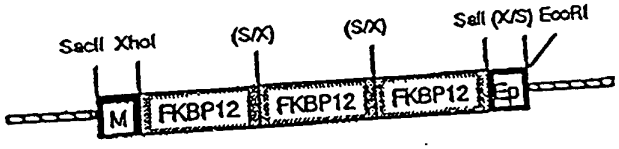


MZF3E



MZE

Cut XhoI/SalI; CIP; + FKBP12 X 3



MF3E

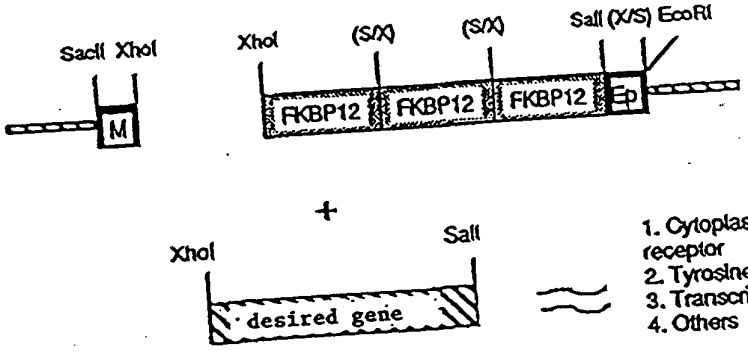


Figure 6B/21

Activity of FK1012A on the chimeric FKBPX3/CD3 zeta receptor

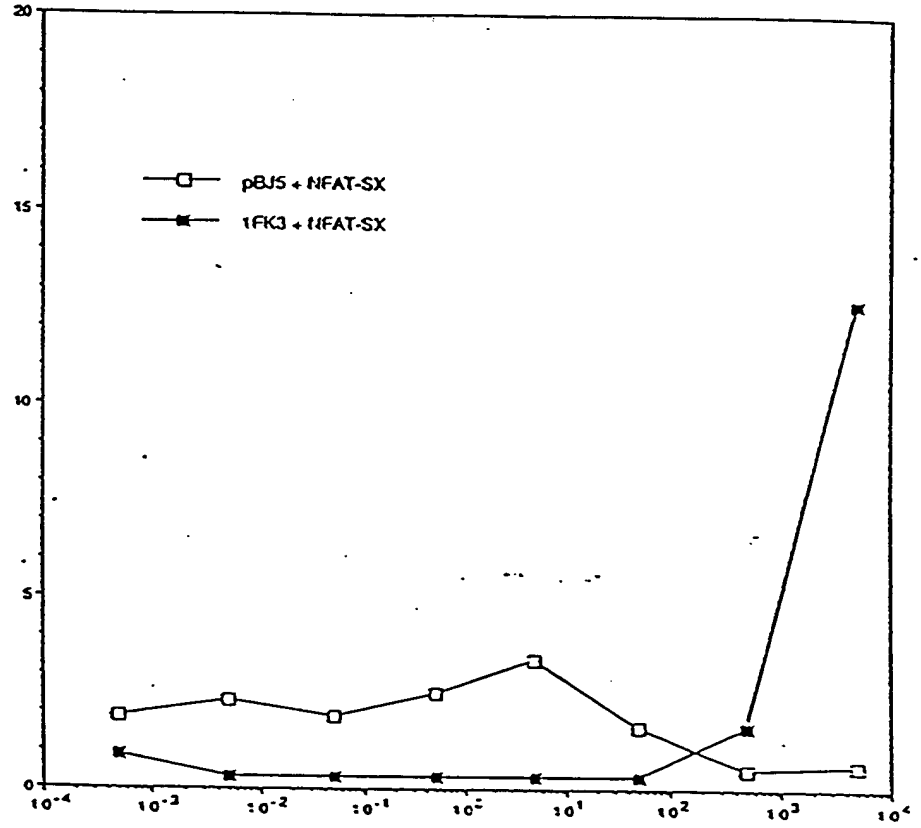


Figure 7/21

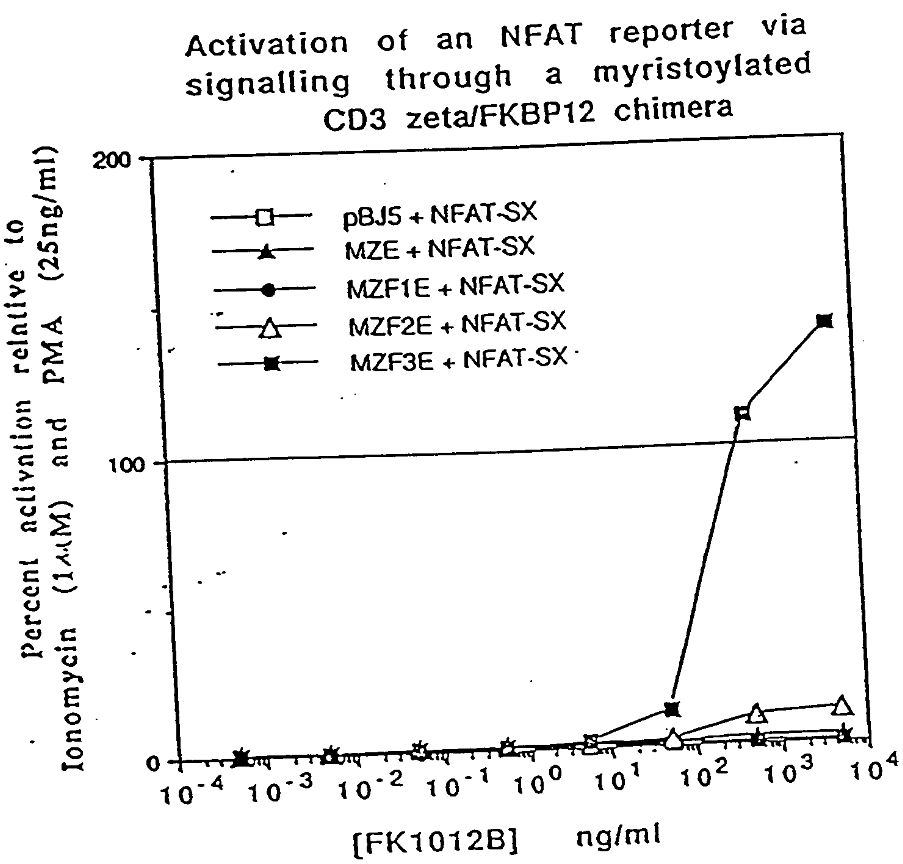

Activation of an NFAT reporter via signalling through a myristoylated CD3 zeta/FKBP12 chimera
pBJ5 + NFAT-SX
MZE + NFAT-SX
MZF1E + NFAT-SX
MZF2E + NFAT-SX
MZF3E + NFAT-SX
Percent activation relative to Ionomycin (1μM) and PMA (25ng/ml)
200
100
0
10^-4 10^-3 10^-2 10^-1 10^0 10^1 10^2 10^3 10^4
[FK1012B] ng/ml

Figure 8/21

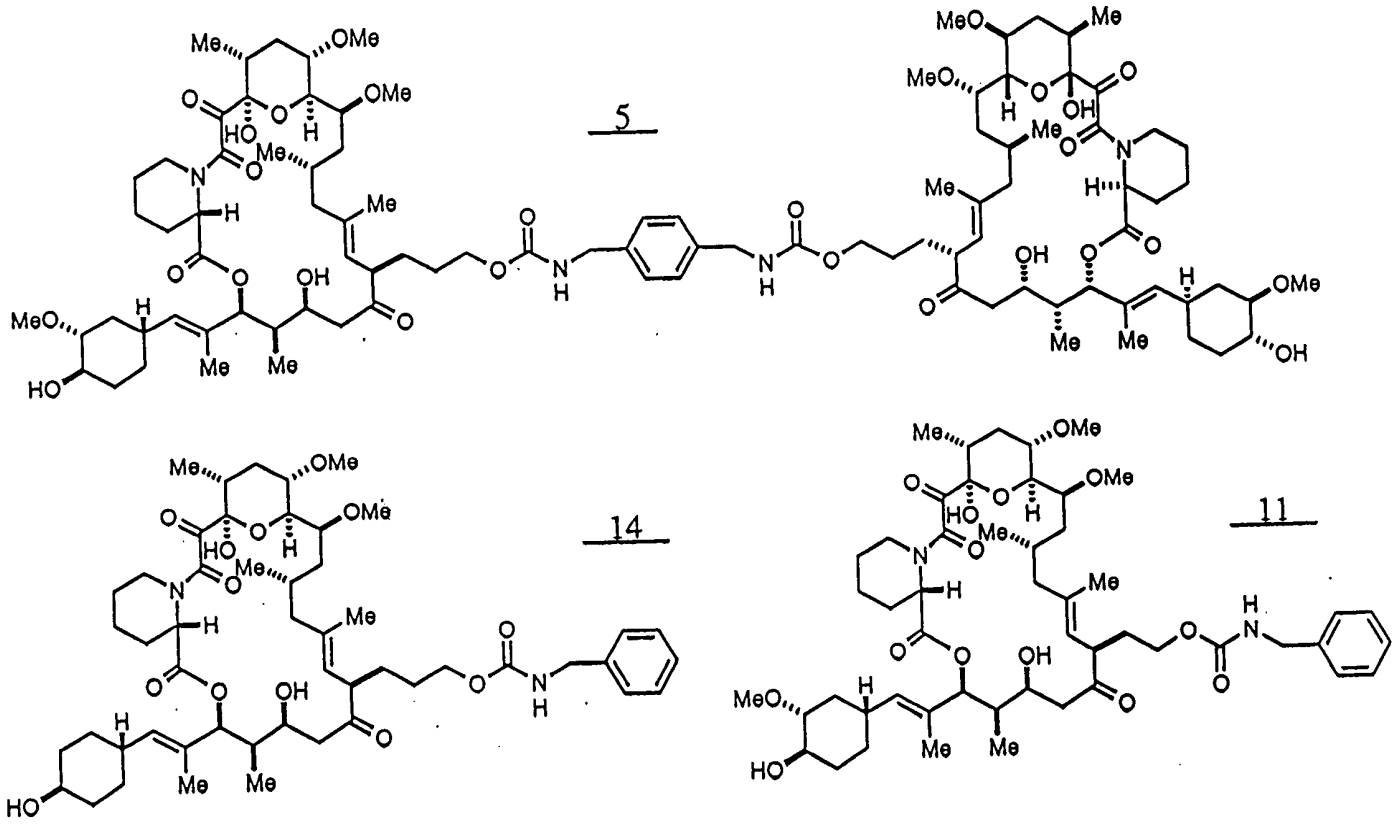
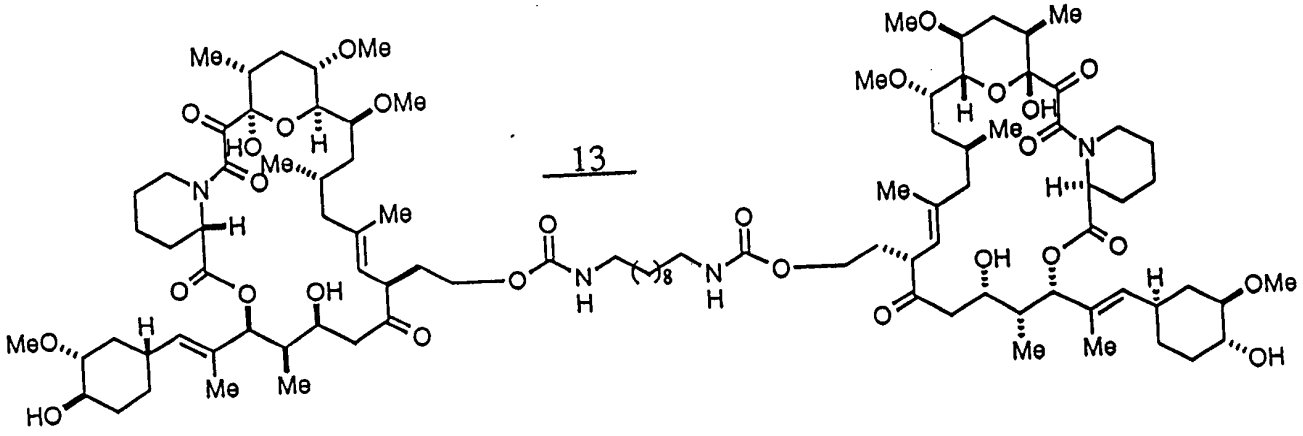
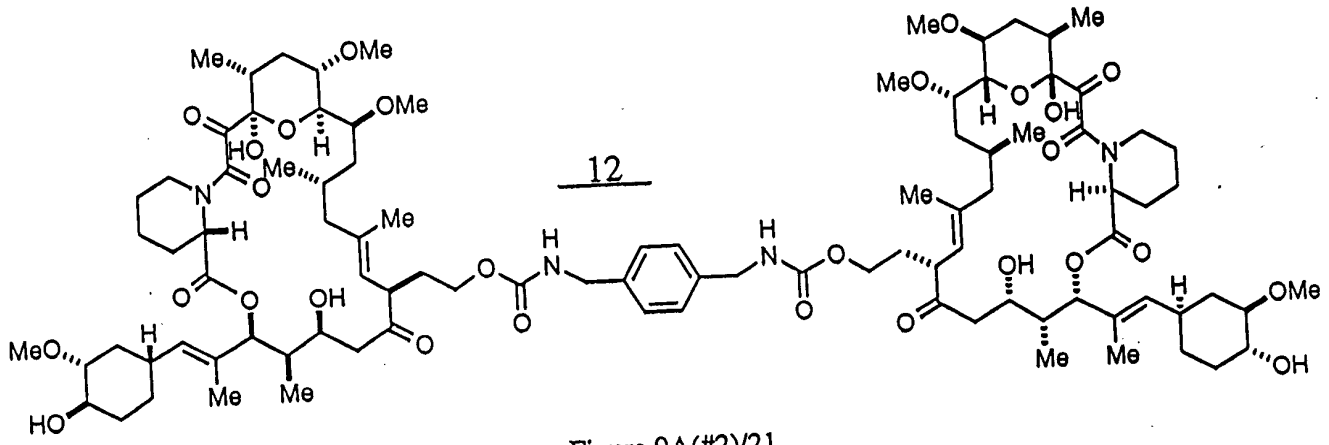


Figure 9A (#1)/21



13



12

Figure 9A(#2)/21

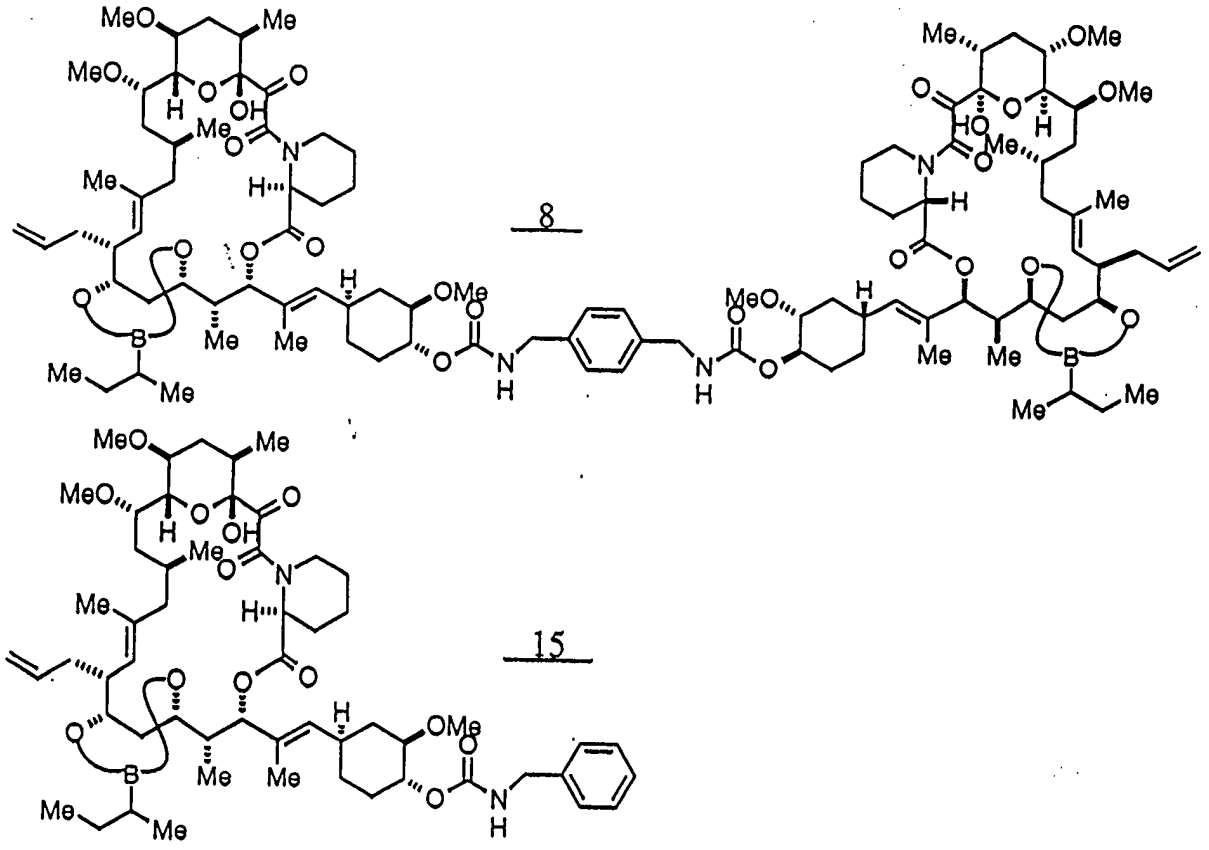


Figure 9B (#1)/21

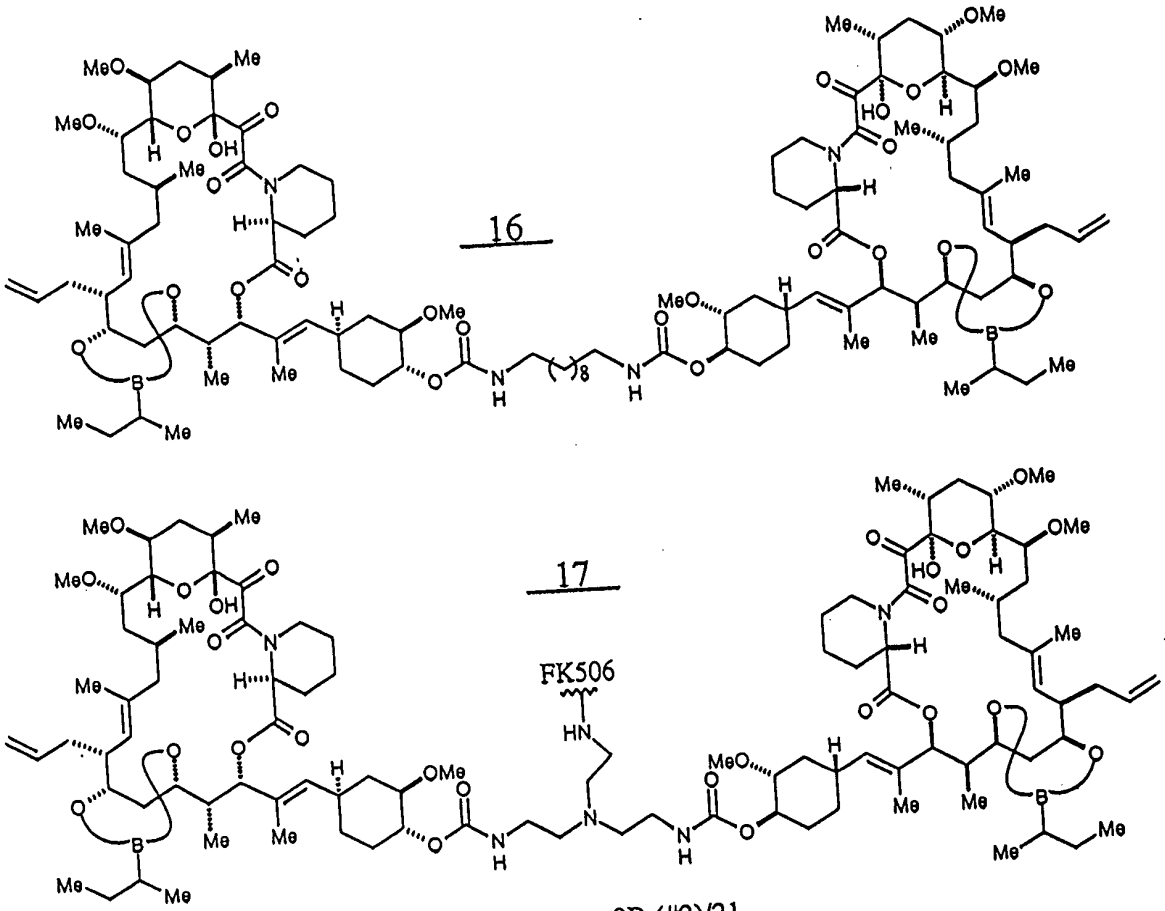


Figure 9B (#2)/21

Scheme 1

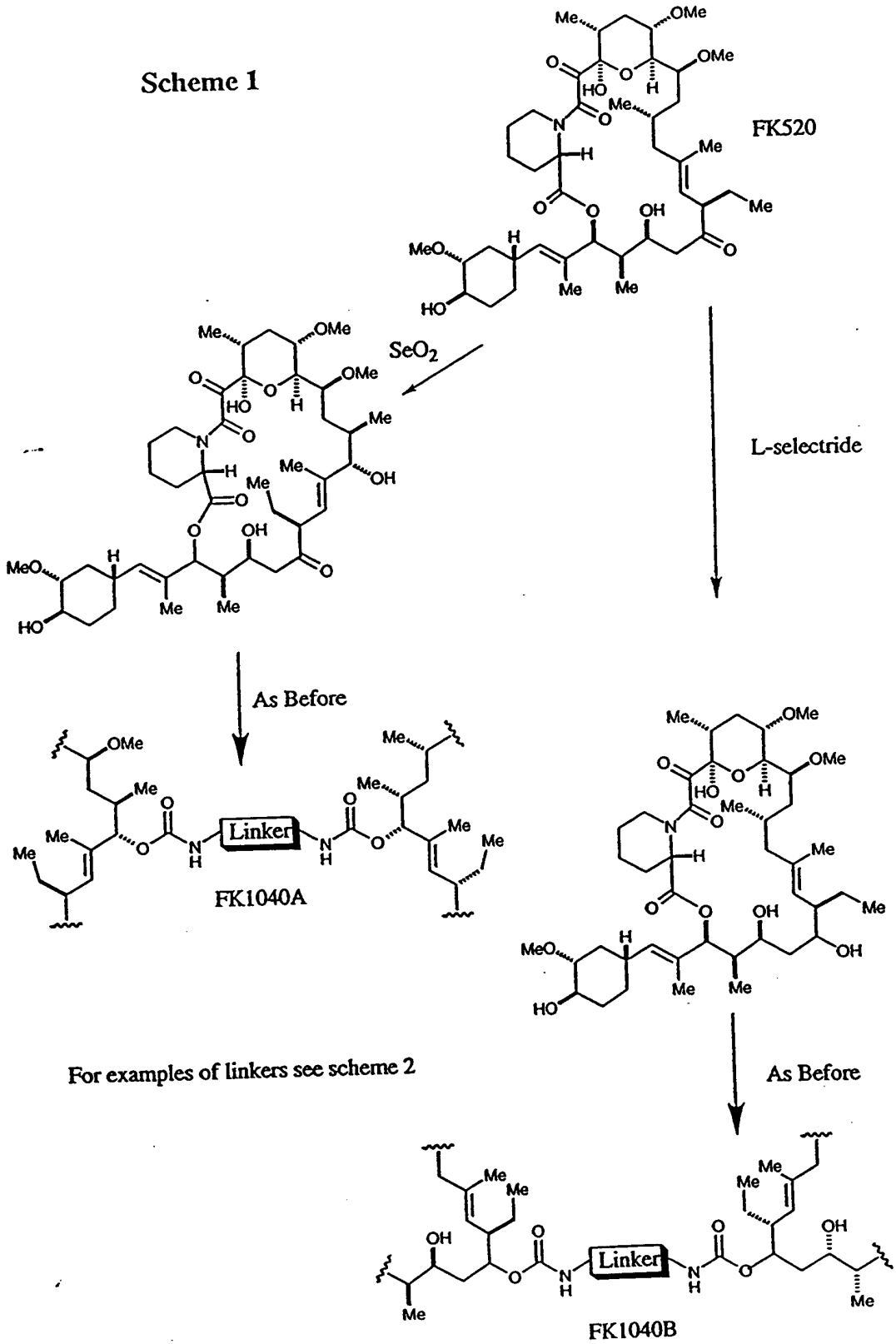
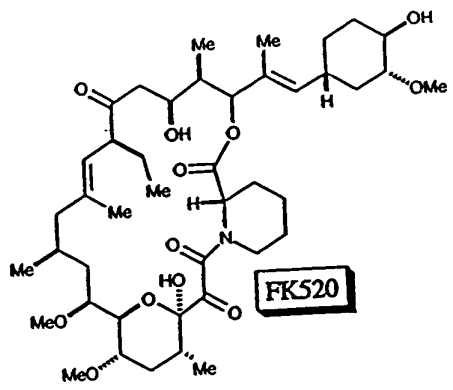
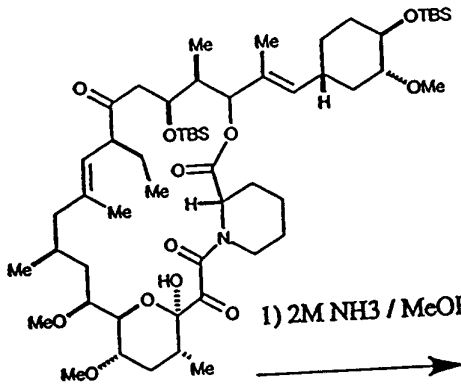


Figure 10/21

Scheme 2: Synthesis of Dimers



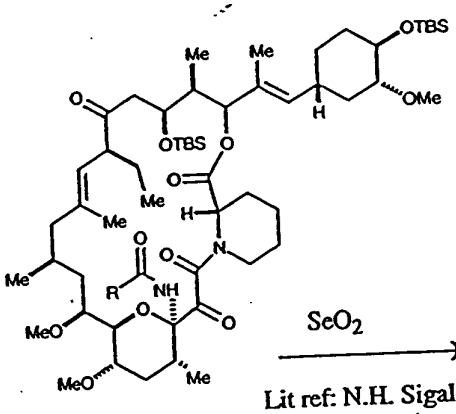
TBS-OTf



1) 2M NH3 / MeOH

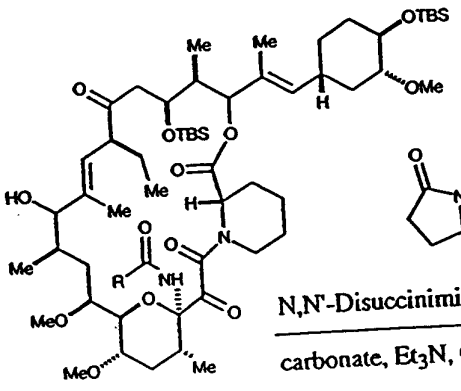
2) formic acid / acetic anhydride or acetic anhydride

Lit refs: D.K. Donald et.al. Tetrahedron Letters p1375, 1991, P.Kocovsky, Tetrahedron Letters p5521, 1992



SeO_2

Lit ref: N.H. Sigal & F.J. Dumont, Ann. Rev. Immunol. p519 1992



N,N'-Disuccinimidyl carbonate, Et_3N, CH_3CN

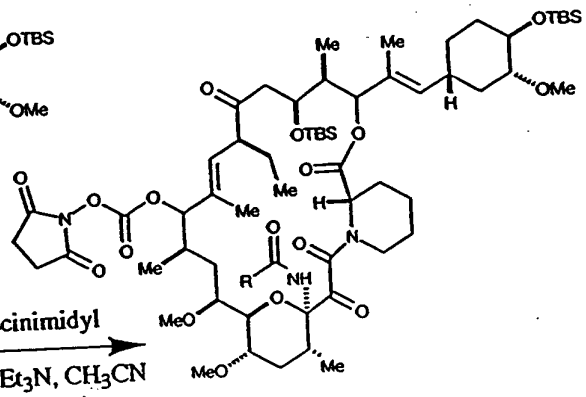
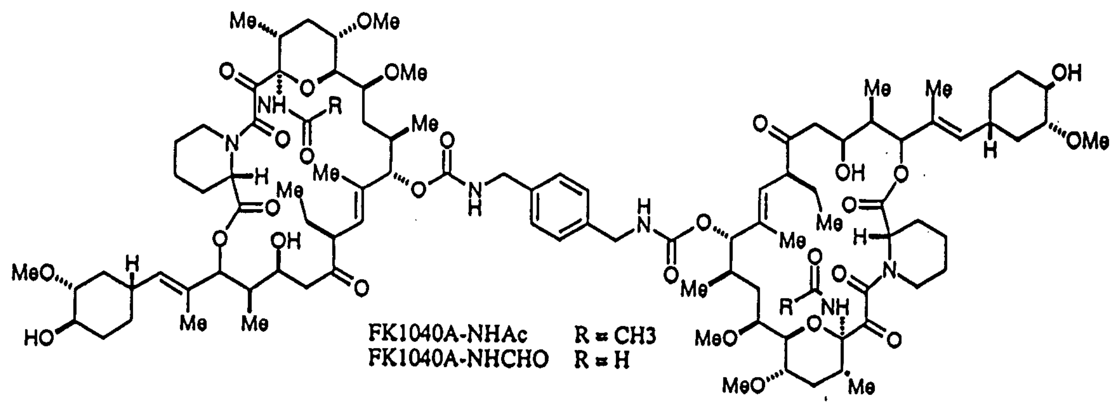
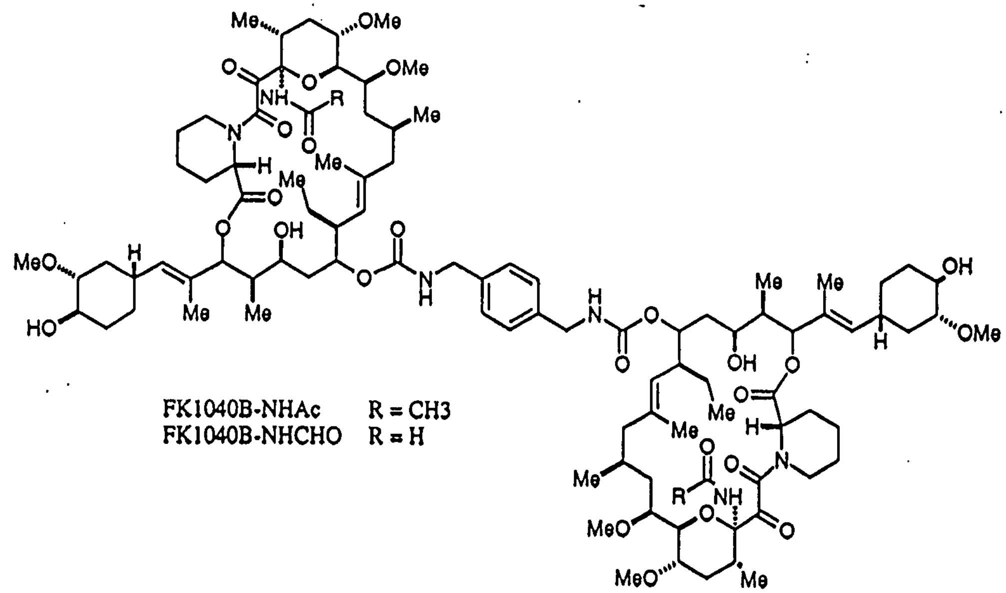


Figure 11A/21



FK1040A-NHAc R = CH3
FK1040A-NHCHO R = H



FK1040B-NHAc R = CH3
FK1040B-NHCHO R = H

Figure 11B (#1)/21

An additional modified FK520 (FK1040) that interferes with FKBP12 yet should bind the FKBP12 mutant: F36A or F99A or Y26A, or combinations thereof is

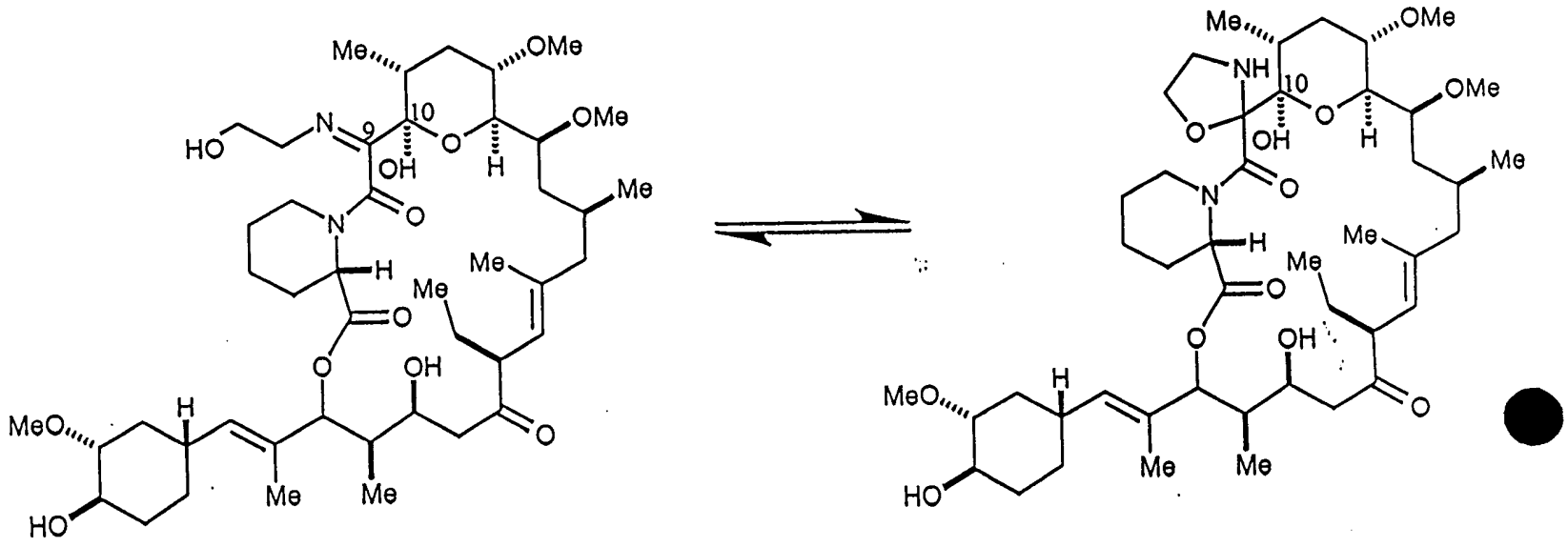


Figure 11B (#2)/21

Scheme 3 Heterodimerization

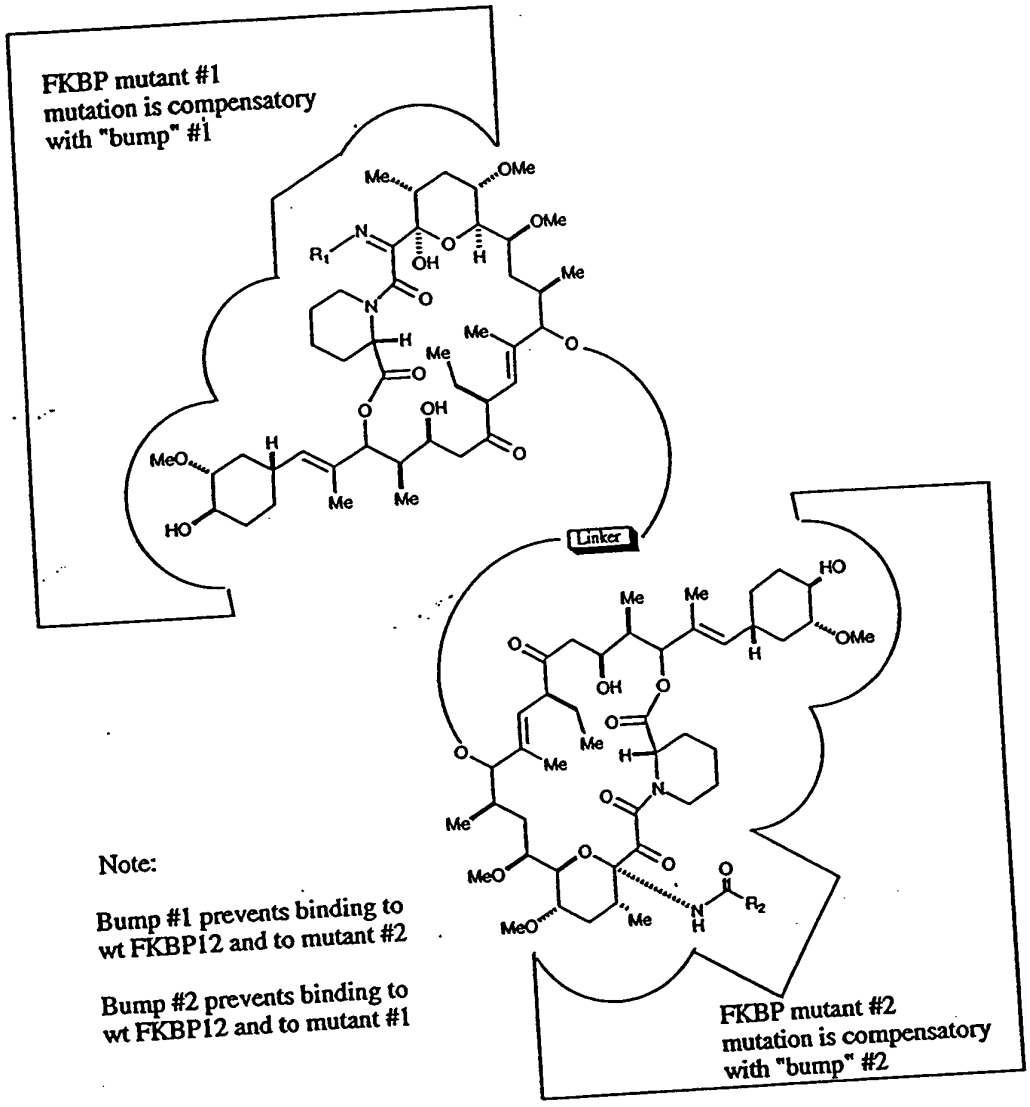
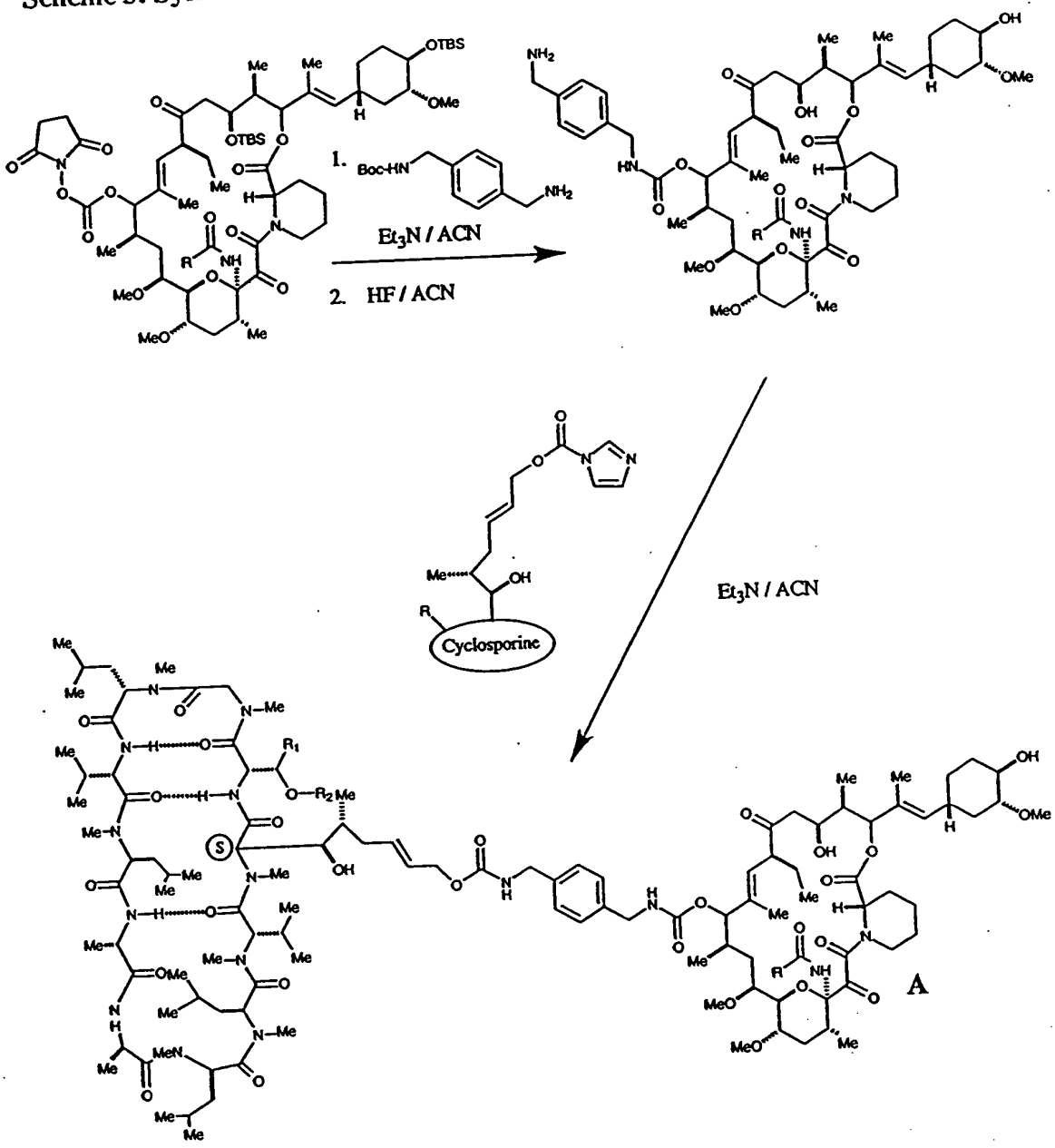


Figure 12/21

Scheme 3: Synthesis of heterodimers



In this example, a heterodimer of a cyclosporine analog and FK520A-NHCO-R were heterodimerized. However, the scheme can easily incorporate other FK506/520 derivatives to form hetero or homodimers

Figure 13/21

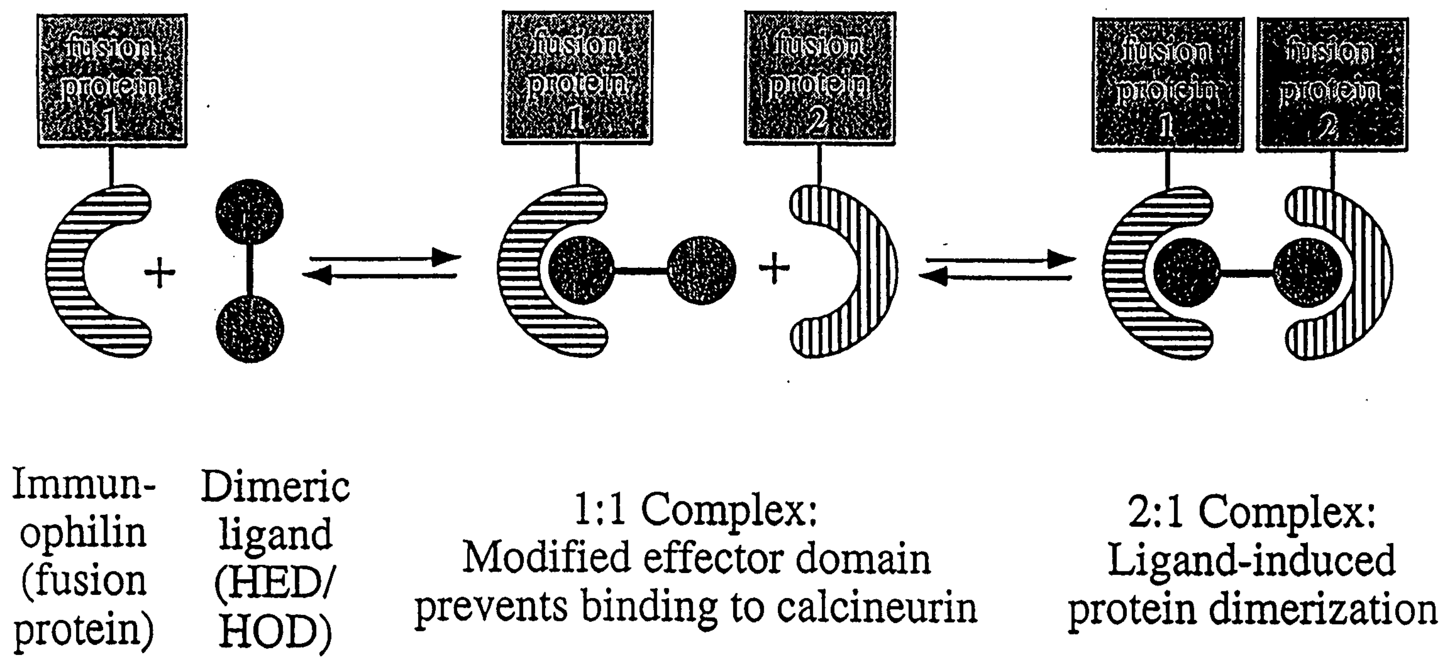


Figure 14/21

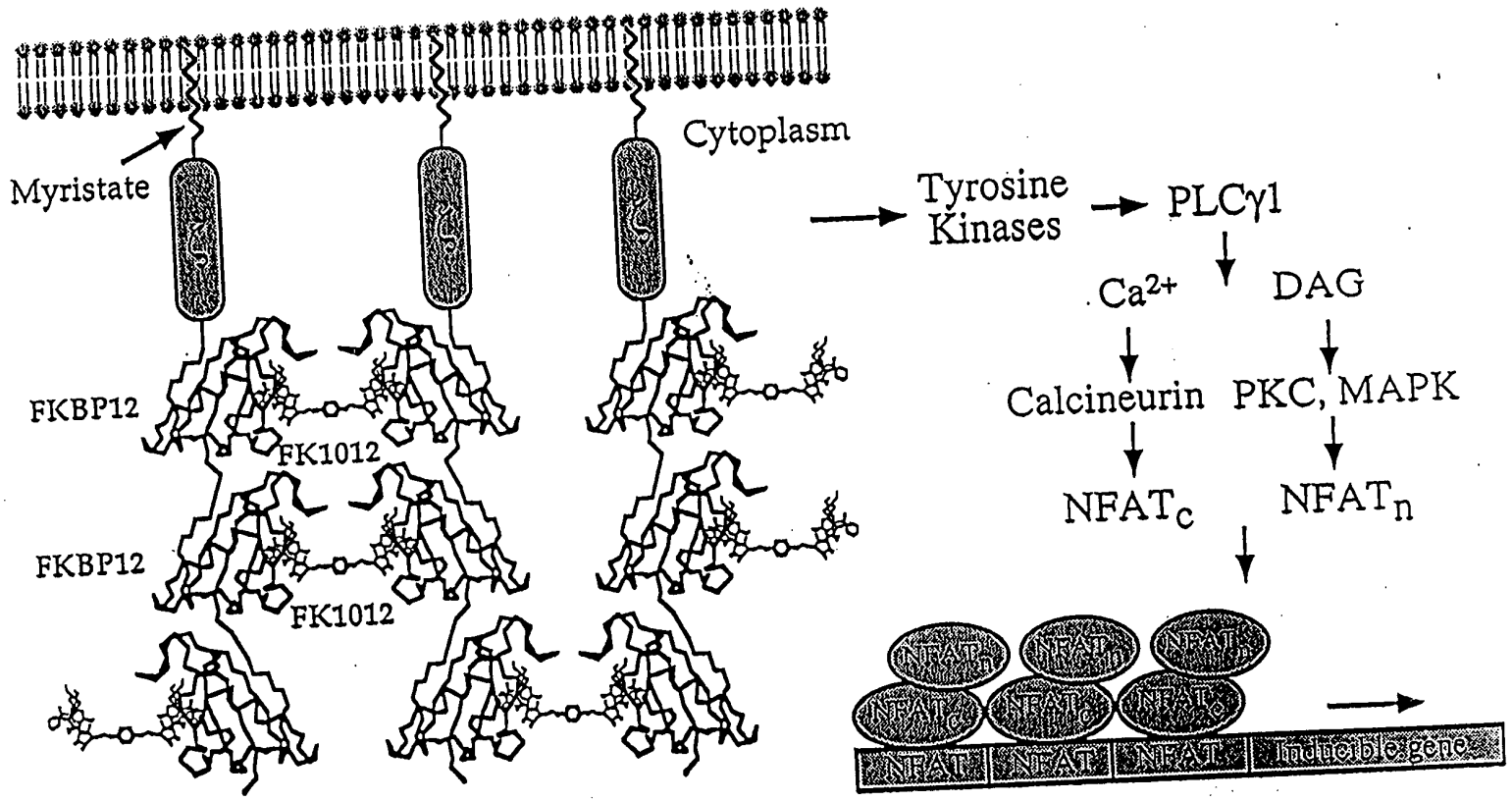
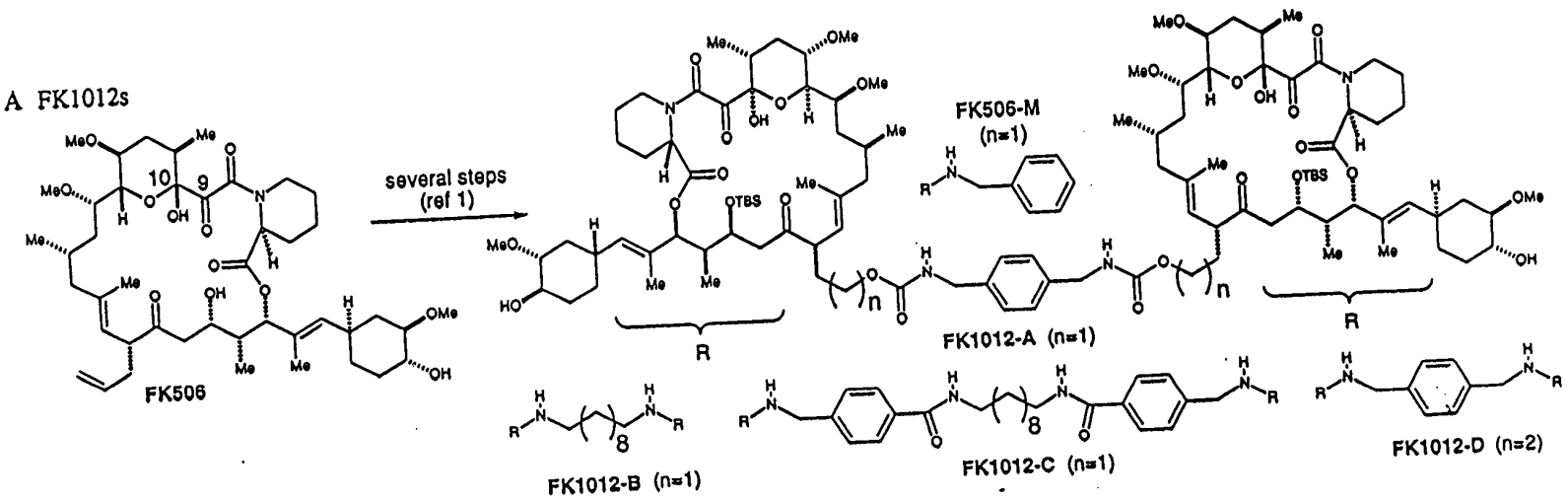

Cytoplasm
Myristate
FKBP12
FK1012
FKBP12
FK1012
Tyrosine Kinases
PLCγ1
Ca2+
DAG
Calcineurin
PKC, MAPK
NFATc
NFATn
NFAT
NFAT
NFAT
Inducible gene

Figure 15/21

A FK1012s



B FK506 Monomer with a C10 Bump

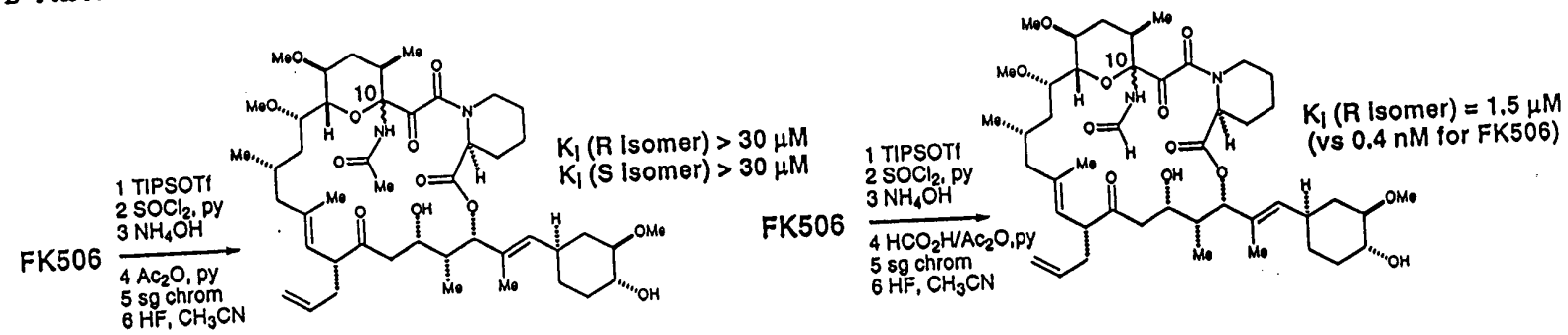
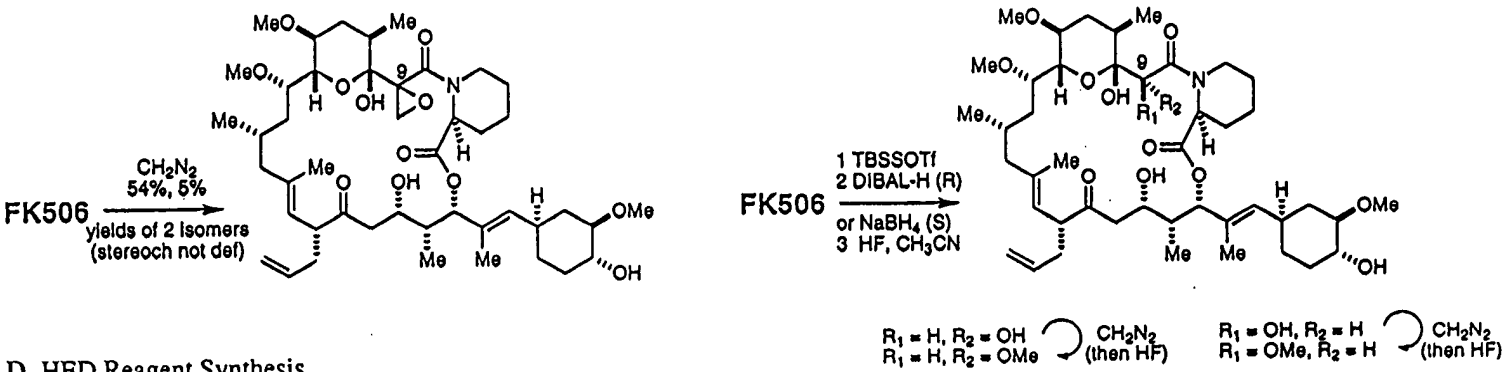


Figure 16 (#1)/21

C FK506 Monomer with a C9 Bump



D HED Reagent Synthesis

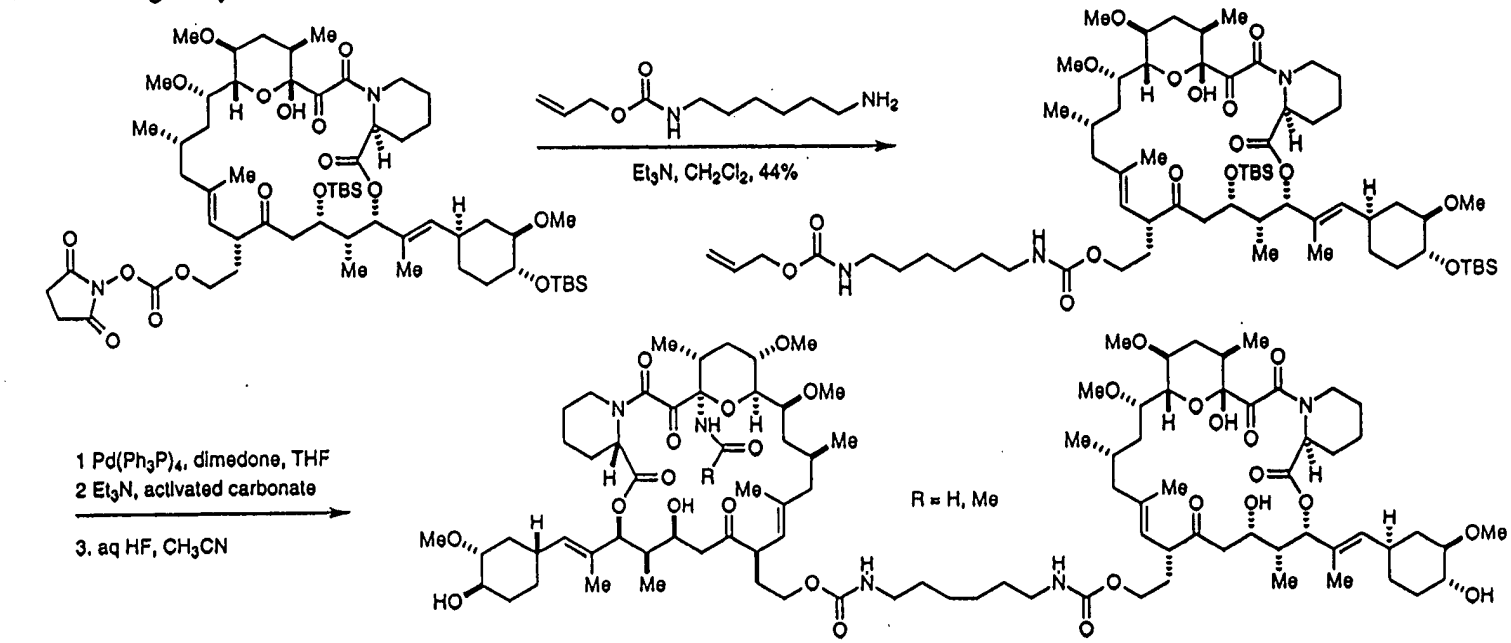


Figure 16 (#2)/21

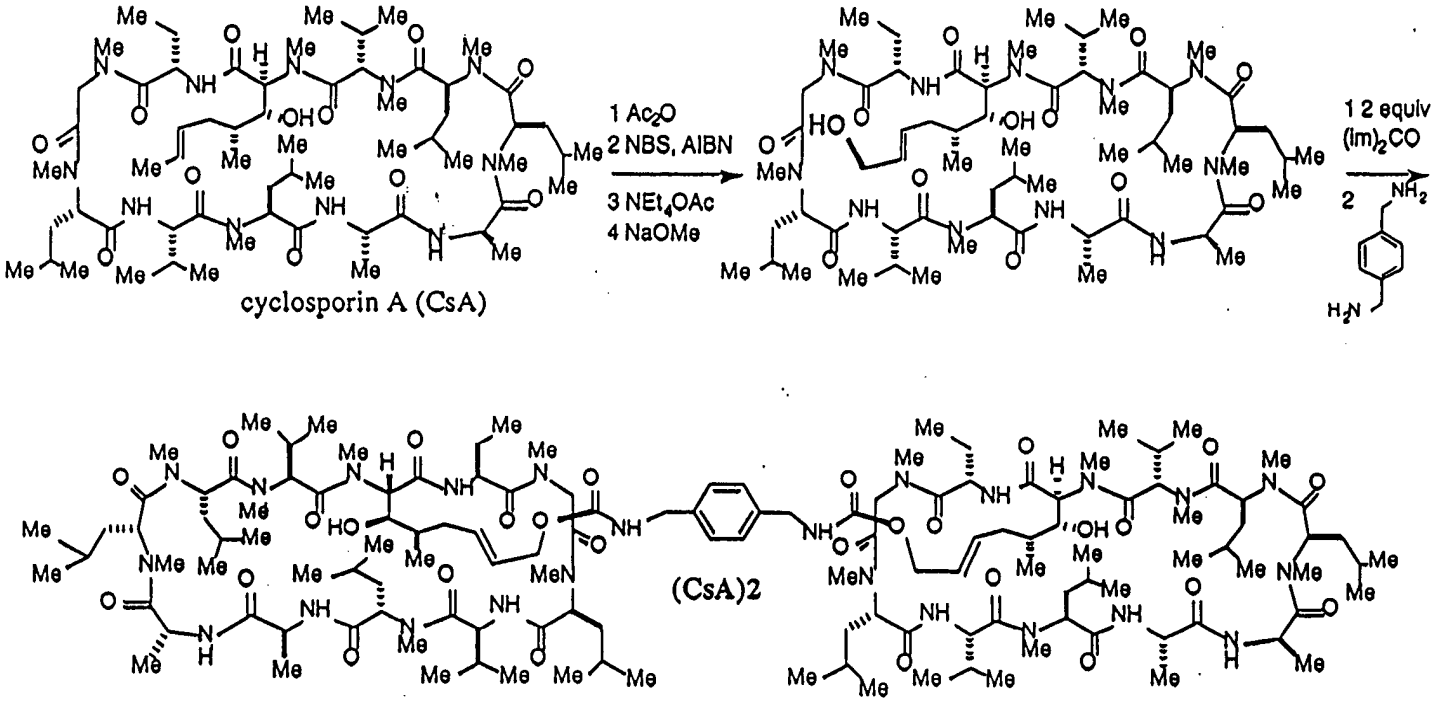

1 Ac2O
2 NBS, AIBN
3 NEt4OAc
4 NaOMe
1 2 equiv
(Im)2CO
2
NH2
H2N
cyclosporin A (CsA)
(CsA)2

Figure 17/21

A cDNA construct

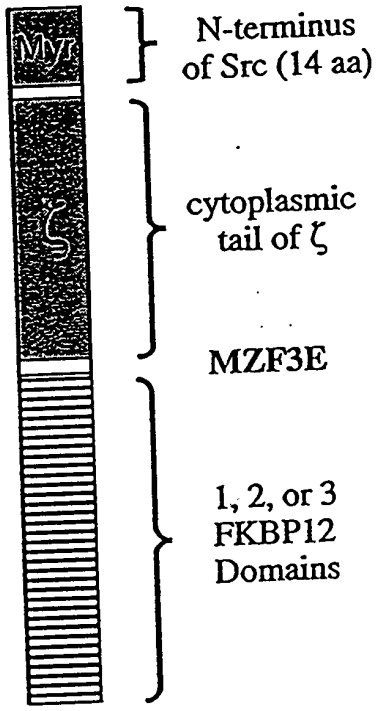


Figure 18A/21

B expressed protein

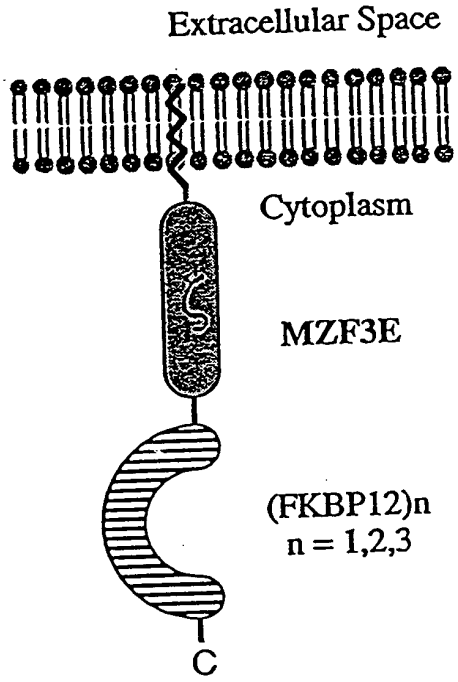


Figure 18B/21

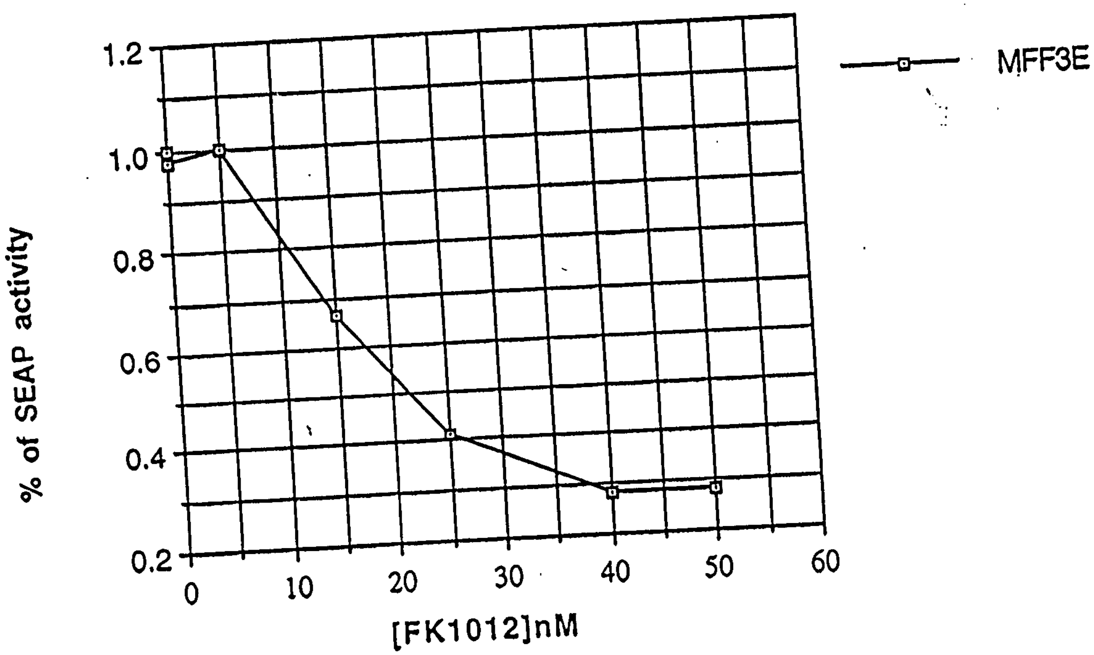

% of SEAP activity
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
[FK1012]nM
MFF3E

Figure 19/21

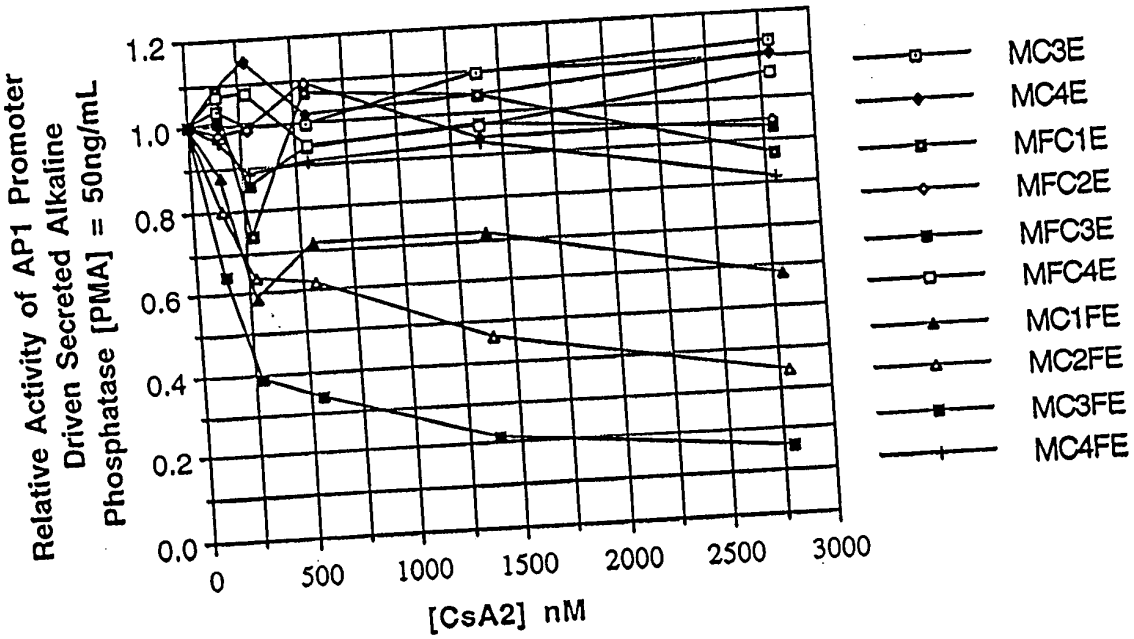


Figure 20A/21

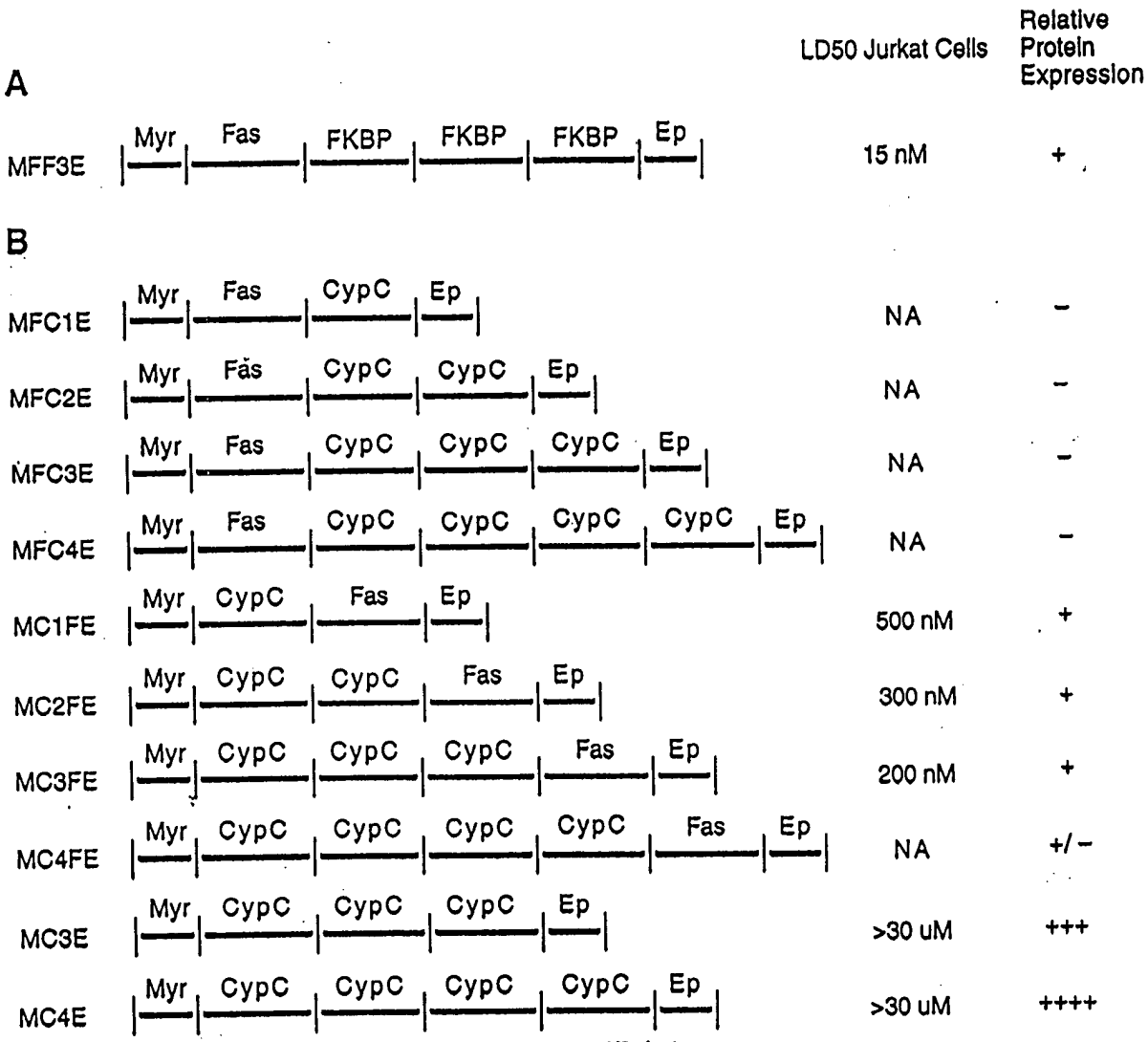


Figure 20B/21

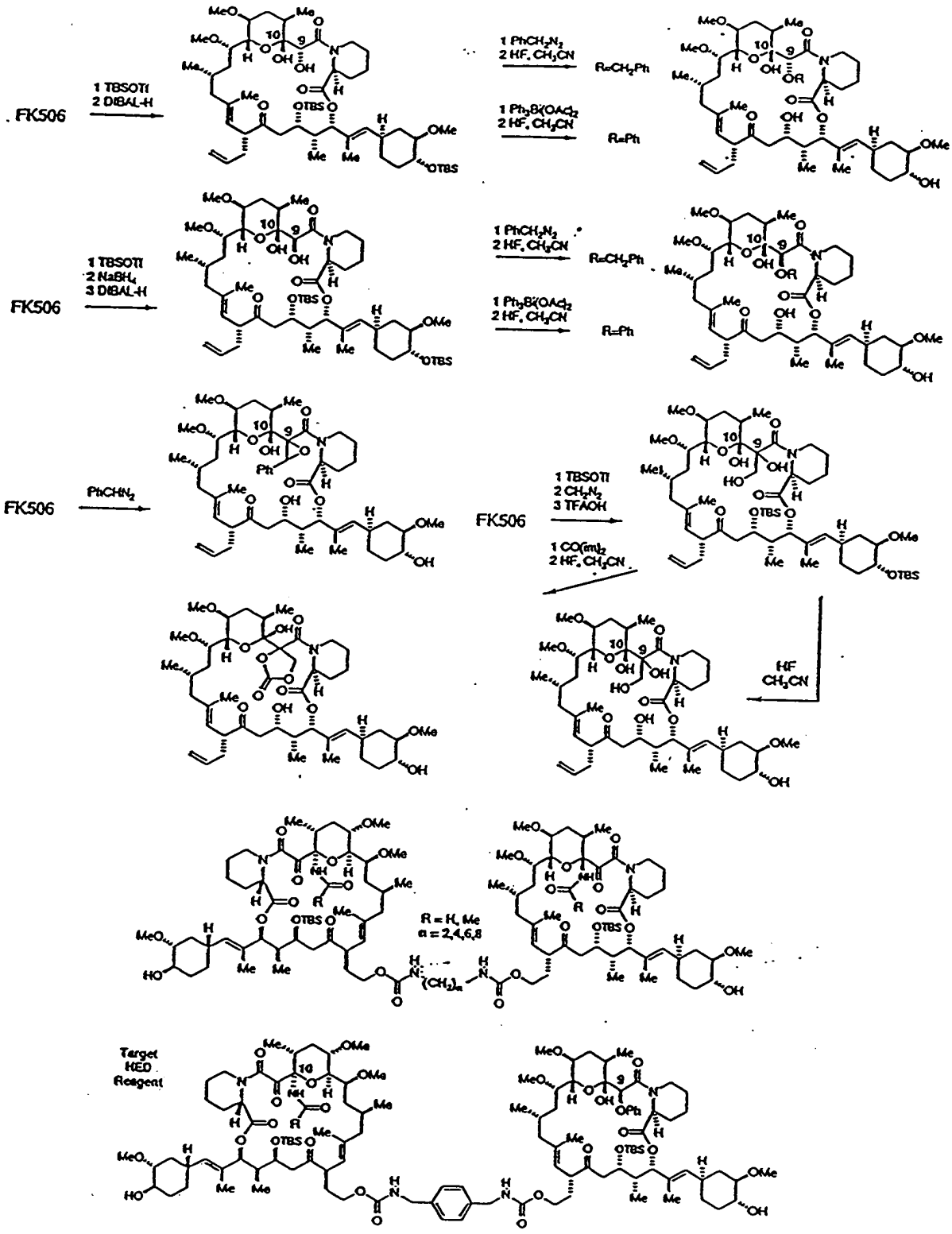

FK506
1 TBSOTf
2 DIBAL-H
1 PhCH2N2
2 HF, CH3CN
R=CH2Ph
1 Ph3Bi(OAc)2
2 HF, CH3CN
R=Ph
1 TBSOTf
2 NaBH4
3 DIBAL-H
PhCHN2
1 TBSOTf
2 CH2N2
3 TFAOH
1 CO(Im)2
2 HF, CH3CN
HF
CH3CN
R = H, Me
n = 2,4,6,8
Target
MED
Reagent

Figure 21/21